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Short Communications

Blunt-end and single-strand ligations by *Escherichia coli* ligase: influence on an in vitro amplification scheme

(Recombinant DNA; T4 ligase; in vitro nucleic acid amplification; nick closing; blunt-end ligation)

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SUMMARY

A ligase-based, in vitro DNA amplification system (LAR) has been described by Wu and Wallace [Genomics 4 (1989) 560–569]. This strategy is based on the ability of a DNA ligase to join the 5′ phosphate of one DNA molecule to the 3′ hydroxyl of a second during a nick-closing reaction. *Escherichia coli* DNA ligase has been used in place of the T4 DNA ligase in our study in order to limit template-independent ligation activities, which lower the sensitivity of this amplification procedure. The results of this study indicate that *E. coli* ligase also joins blunt-ended DNA molecules and some single-stranded oligodeoxyribonucleotides, in the absence of a complementary template, with an efficiency which is sensitive to both the concentrations of DNA substrate and enzyme.

INTRODUCTION

Methods devised for the in vitro amplification of nucleic acids have had profound impacts on such diverse areas as molecular biology, forensic medicine, evolution and developmental biology (Erlich, 1989). The polymerase chain reaction (PCR; Saiki et al., 1985; Mullis and Faloona, 1987) and a transcription-based amplification system (TAS; Kwoh et al., 1989) are two in vitro amplification strategies that have been developed which produce their amplified products by DNA replication and RNA transcription, respectively. Most recently, a ligase-based strategy, LAR, has been presented by Wu and Wallace (1989a) for amplifying nt sequences. This strategy consists of annealing two oligos to a common complementary target sequence such that the 5′ phosphate of one of the oligos (donor oligo) is adjacent to the 3′ hydroxyl of the second oligo (acceptor oligo). DNA ligase is then used to join the two oligos in a nick-closing reaction. When the product of ligation generated in the first cycle is subjected to succeeding cycles of denaturation, annealing and ligation in the presence of complementary donor and acceptor oligos, amplification of the original target sequences is carried out in an exponential manner. As originally indicated by Wu and Wallace (1989a), the one criterion determining the feasibility of this LAR system depends on the extent to which the donor and acceptor oligos are joined by template-independent processes. Such template-independent, blunt-end ligation reactions at the outset of the LAR would render

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Abbreviations: AP, alkaline phosphatase; β-NAD⁺, β-nicotinamide adenine dinucleotide; bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; *Ec* ligase, *E. coli* DNA ligase; HPLC, high-performance liquid chromatography; LAR, ligation amplification reaction; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PA, polyacrylamide; PCR, polymerase chain reaction; PEG, polyethylene glycol; pmol, picomoles; ss, single strand(ed); 1 × T buffer, see Fig. 2, legend; TAS, transcription-based amplification system; TE, 10 mM Tris·HCl pH 7.4/1 mM EDTA; u, unit(s).

this amplification method highly insensitive. Wu and Wallace (1989a) explored the use of T4 DNA ligase in the LAR and described conditions which suppress such blunt-end ligation. We have extended this study by using *Ec* ligase. This enzyme has been reported to possess the ability to discriminate against the joining of blunt-ended molecules (Sugino et al., 1977) except in the presence of volume-exclusion agents such as polyethylene glycol (PEG) or Ficoll (Zimmerman and Pheiffer, 1983) and with monovalent cations such as Na^+ or K^+ (Hayashi et al., 1985). Our results indicate that *Ec* ligase possesses both blunt-end and ss ligase activities in the absence of volume-exclusion agents and, like T4 DNA ligase, presents serious problems for the successful application of the LAR strategy to the amplification of low-copy-number nucleic acid targets.

EXPERIMENTAL AND DISCUSSION

(a) Test for blunt-end ligation reactions

A summary of the LAR strategy first described by Wu and Wallace (1989a) is shown in Fig. 1. To determine whether *Ec* ligase possesses blunt-end activity in the absence of volume-exclusion agents, the following experiment was performed. Donor ^{32}P-labeled 20-nt oligos 88–100.1 and 88–140.1 were annealed to their complementary unlabeled 20-nt acceptor oligos 88–101.2 and 88–139.1 and then ligated with either *Ec* ligase or T4 DNA ligase in an attempt to form a 40-bp product. *Ec* ligase is capable of producing an AP-resistant, 40-bp DNA fragment (Fig. 2, lanes 3–4), as well as a small quantity of 60-bp fragments by blunt-end ligation. These products were not observed when DNA ligase was omitted from the reaction (lanes 9–10). The formation of AP-resistant, 40-, 60-, 80-, 100- and 120-bp products could be observed after the ligation of the 20-nt oligos by T4 ligase (lanes 11–12). The formation of a 40-nt ss ligation product did not occur in *E. coli* reactions consisting only of contiguous oligo pairs 88–100.1 and 88–101.2 (lanes 5–6) or 88–139.1 and 88–140.1 (data not shown). Similarly, no such ss ligation activity is observed with T4 ligase (lanes 7–8). However, further analysis of *Ec* ligase for ss ligation activity indicated some positive results for certain oligos (see section c). The conditions used for the T4 DNA ligase enabled approximately 22% of the input oligos to be incorporated into AP-resistant, blunt-end ligated products 40 to 120 bp in size. The *Ec* enzyme allowed 0.05% of the input DNA to be converted into an AP-resistant, 40-bp form.

(b) Factors affecting the blunt-end ligation activity of *Ec* ligase

The blunt-end activity observed for *Ec* ligase led us to investigate the conditions employed during the ligation

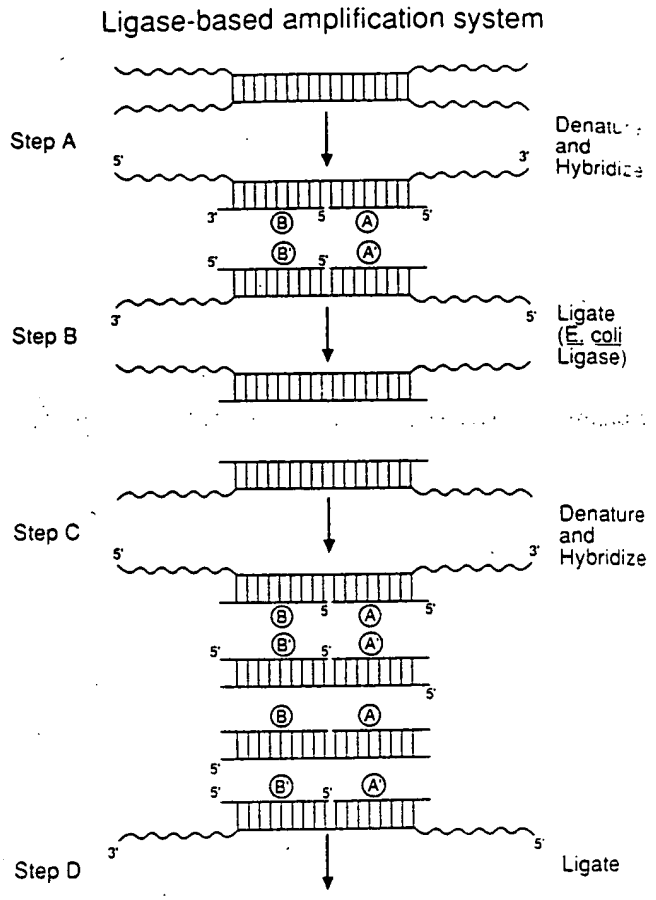


Fig. 1. Schematic diagram of an in vitro amplification system described by Wu and Wallace (1989a). Steps **A** and **B** represent oligos whose sequences are contiguous upon a common target nucleic acid. All oligos were synthesized using the phosphoramidite method in an Applied Biosystems DNA synthesizer (Model 380A) and purified by HPLC using a C8 column and 15% PA electrophoresis. Oligos eluted from PA gels were purified from TE by NENSORB column chromatography according to the directions supplied by the manufacturer. Oligos for ligation reactions were end-labeled with T4 kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). (A') and (B') are oligos complementary to (A) and (B), respectively. In these experiments, these oligos were designed to be homologous to portions of the first 49 nt of the coding sequence, where the first nt is the A residue of the ATG start codon of the human β^A-globin-encoding gene (Lawn et al., 1980). Oligos: 88–101.2, 5'-GACTTCTCCTCAGGAGTCAG; 88–140.1, 5'-CTGACTCC-TGAGGAGAAGTC; 88–100.1, 5'-CCCACAGGGCAGTAACG-GCA; 88–139.1, 5'-TGCCGTTACTGCCCTGTGGG; 88–165, 5'-CTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGT-GGG; 88–175, 5'-ATGGTGCACCTGACTCCTGAGGAGAAG-TCTGCCGTTACTG; 88–99.1, 5'-TCAGGAGTCAGGTGCAC-CAT; 88–102, 5'-CAGTAACGGCAGACTTCTCC.

reactions. Standard ligation reaction conditions for *Ec* ligase consisted of 150 pmol of each oligo, 50 u of ligase, 8 mM Mg^{2+}, and 26 μM β-NAD^+ at 37°C for 10 min. Experiments were conducted to test the effects on blunt-end ligation of varying concentrations of: (a) substrate oligos, (b) ligase, (c) co-factors (β-NAD^+ and Mg^{2+}) and (d) temperature.

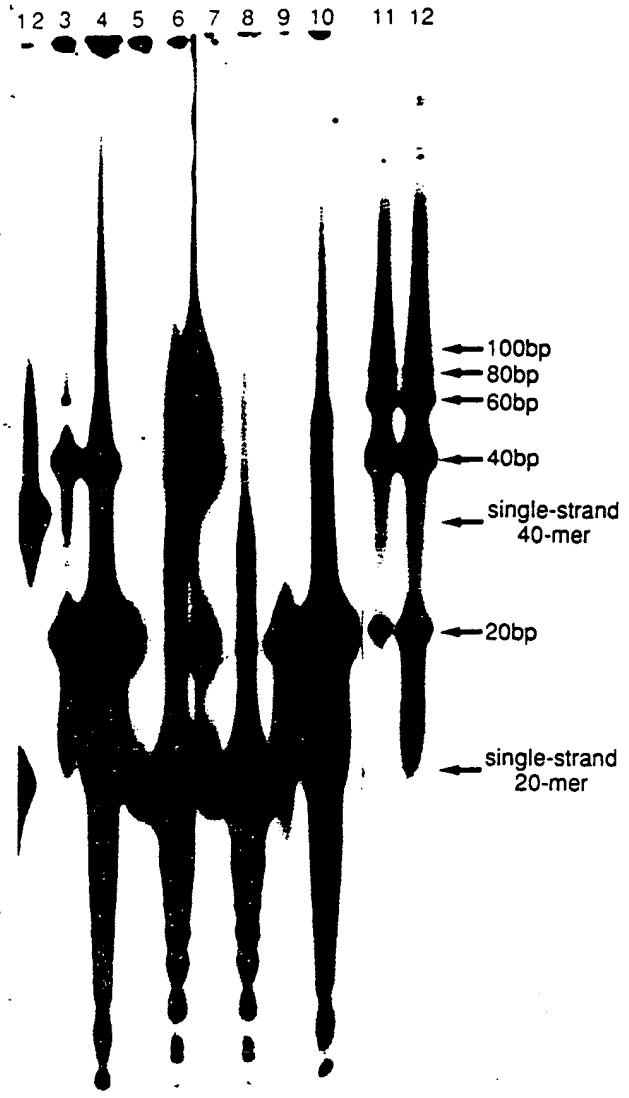


Fig. 2. Demonstration of blunt-end ligation reactions mediated by *Ec* and T4 DNA ligases. 5′ ^{32}P-labeled and purified 20-nt donor oligo (1 μg; about 150 pmol) was mixed with an equal amount of its contiguous, purified, unlabeled, 20-nt acceptor oligo in the presence of 250 fmol of a complementary 40-nt target oligo in a total volume of 25 μl. Prior to the ligation reactions, the oligos were denatured in a batch for 5 min at 100°C and aliquoted to individual tubes. These aliquots of oligos were diluted with an equal volume of 2 × T-buffer (1 × = 40 mM Tris·HCl pH 8.0/8 mM $MgCl_2$/25 mM NaCl/2 mM spermidine/5 mM DTT/80 μg per ml BSA), permitted to anneal at 60°C for 10 min and then cooled to 37°C. The ligation reactions were adjusted to a total volume of 60 μl by the addition of 10 μl of ligase (50 u of either *Ec* or T4 ligase), co-factor (β-NAD^+ to a final concentration of 26 μM for the *Ec* enzyme or ATP to 2 mM for the T4 DNA ligase), and 3 μg of *Hin*dIII-cut λ DNA (as an internal ligation control) in 1 × T-buffer. The ligation reactions were incubated for 30 min and then terminated by heating at 60–65°C for 10 min. Each ligation reaction was analyzed using a AP-protection assay (Sugino et al., 1977; Weiss et al., 1968).

Blunt-end ligase activity was monitored by using 150 pmol of ^{32}P-labeled oligos 88–100.1 and 88–140.1 which were mixed with equal amounts of complementary oligos 88–101.2 and 88–139.1 in a total volume of 25 μl, with no complementary target or control λ DNA. Denaturations, annealings and ligations were accomplished as for a single cycle of an amplification (see below), except that 20 μl of each reaction were placed in a tube containing 1 μg of *Hin*dIII-digested λ DNA after the addition of the enzyme to the reaction to serve as a parallel control for ligase activity. The blunt-end activities of *Ec* and T4 ligases were quantitated by excising the appropriate regions of the polyacrylamide gel containing the AP-treated reaction products and calculating the percent radioactivity associated with the ligation products based on the amount of labeled oligo used in the reaction.

Lanes **1** and **2** show the electrophoretic mobility of ss oligos (20 and 40 nt, respectively) in a non-denaturing, 15% PA. Lanes **3** and **4** show the products of a blunt-end ligation reaction catalyzed by the *Ec* enzyme with and without subsequent AP treatment, respectively. Lanes **5** and **6** are *Ec* ligase-mediated reactions containing the contiguous oligos 88–101.2 and 88–139.1, with or without AP treatment, respectively. Lanes **7** and **8** are identical to **5** and **6** except that T4 ligase was used. Lanes **11** and **12** are identical to **3** and **4**, respectively, except that the T4 enzyme was used. Lanes **9** and **10** are identical to **3** and **4**, but without the addition of any ligase.

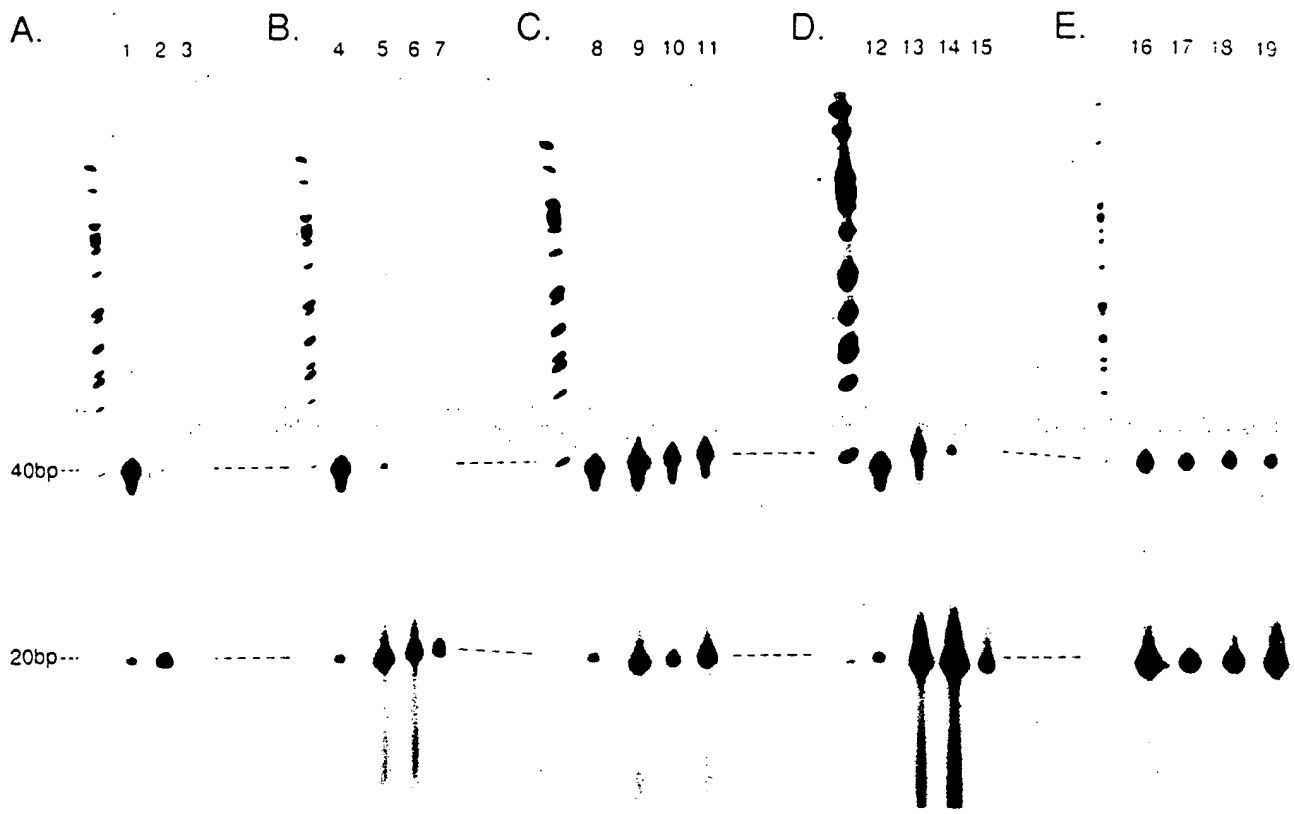
The amounts of blunt-end ligation using 150 (1 μg), 45 (0.3 μg) and 15 (0.1 μg) pmoles of substrate are presented in Fig. 3A. Interestingly, the efficiency of blunt-end ligation decreased by more than 25- and 50-fold with a 1/3 and 1/10 decrease in substrate concentration. No detectable blunt-end ligation activity was observed after 10 min of ligation using 15 pmol of oligo substrate.

Decreasing the amount of *Ec* ligase used in each reaction resulted in a pronounced reduction in blunt-end ligase activity (Fig. 3B). A fivefold reduction in enzyme activity produced a 14-fold reduction in the formation of the blunt-end ligation products. Reduction of *Ec* ligase to 1 and 0.1 u per reaction yielded no detectable 40-bp blunt-end products. Importantly, this reduction in blunt-end ligation activity was not paralleled by an impairment of the ability of *Ec* ligase to ss nicks (data not shown).

Ec ligase is known to function through an intermediate consisting of β-NAD^+ attached to the enzyme. However, the titration experiment shown in Fig. 3C reveals that the efficiency of both blunt-end ligation and nick-closing activity are not affected by β-NAD^+ concentration over the range 0–26 μM, since there appears to be sufficient β-NAD^+ carried with the *Ec* ligase. However, as the amounts of Mg^{2+} in each ligation reaction were titrated from 8, 4, 1 and 0 mM, the amount of 40-bp blunt-end ligation product decreased proportionally (Fig. 3D). The reduction of Mg^{2+} from the nick-closing ligation also prominently decreased the overall activity of *Ec* ligase (data not shown).

Increasing the temperature at which blunt-end joining mediated by *Ec* ligase occurred resulted only in an approximate threefold reduction in the formation of the 40-bp blunt-end product (Fig. 3E). While the increase in the temperature of the ligation reaction parallels a decrease in blunt-end ligation activity, it also parallels a concomitant decrease in nick-closing ligation activity (data not shown).

As indicated previously, lowering the substrate oligo to



A. Oligonucleotide Substrate				B. Enzyme Concentration				C. β-NAD$^+$ Concentration				D. Mg^{++} Concentration				E. Temperature			
Substrate (pmol.)	150	45	15	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150
β-NAD$^+$ (μM)	26	26	26	26	26	26	26	26	2.6	1.0	0	26	26	26	26	26	26	26	26
Mg^{++} (mM)	8	8	8	8	8	8	8	8	8	8	8	8	4	1	0	8	8	8	8
E. coli ligase (u)	50	50	50	50	10	1	0.1	50	50	50	50	50	50	50	50	50	50	50	50
Amount of 40 bp product (fm)	57	2	ND	57	4	ND	ND	57	48	26	20	57	46	9	2	69	44	41	25
Temp. (°C)	37	37	37	37	37	37	37	37	37	37	37	37	37	37	37	16	23	37	42

Fig. 3. Autoradiograph of titration experiments examining the constituents of blunt-end ligation reactions. Panels **A–E** represent the results of titrating out substrate oligos, *Ec* ligase, β-NAD$^+$, Mg^{2+} and different incubation temperatures, respectively. Lanes 1, 4, 8, 12, and 18 of panels **A–E** are the ligation products obtained under the standard conditions (see Fig. 2). Each reaction was resolved in a non-denaturing, 15% polyacrylamide gel after AP treatment. All components and conditions are listed below each lane. ND, not detectable.

15 pmol and using 50 u of *Ec* ligase resulted in no detectable blunt-end ligation product (Fig. 3A). Interestingly, using a combination of lower substrate (15 pmol) and ligase activity (10 or 1 u) also resulted in little or no loss in nick-closing activity, while maintaining no detectable blunt-end ligation activity (data not shown). Consequently, the optimal concentrations of substrate and ligase needed to achieve maximal nick-closing activity with minimal blunt-end activity is 10–1 u of ligase and 15 pmol of substrate.

(c) The ss activity of ligase

The capability of *Ec* ligase to join two non-complementary ss oligos together was studied in some detail. As indicated previously, when oligo pairs 88–100.1 and 88–139.1 were phosphorylated and ligated to oligos 88–101.2 and 88–140.1, respectively, no target-independent ss ligation activity was observed (Fig. 4, lanes 1, 2). However, when oligo 88–101.2 was used as the donor oligo and 88–100.1 was the acceptor, a target-independent ss AP-resistant ligation product was observed (lane 3). The formation of this AP-resistant product was dependent on the presence of *Ec* ligase (lane 4). Similarly, when a second set of contiguous oligos, 88–99.1 (donor) and 88–102, was used in another target-independent ligation experiment, a 40-nt AP-resistant product was observed (lane 5). Interestingly, when the 88–99.1 oligo was placed in the ligation reaction alone, it was capable of self-ligation (lane 6); this self-ligation was also noted for oligo 88–101.2 (lane 7). T4 ligase was observed to generate the same AP-resistant ligase products as *Ec* ligase (lane 8).

(d) Ligase-based amplification strategy

An LAR scheme is depicted in Fig. 1. The results of our experiments on *Ec* ligase suggested that blunt-end and ss. target-independent ligation activities could be controlled by reducing the substrate oligos to 15 pmol or less and reduc-

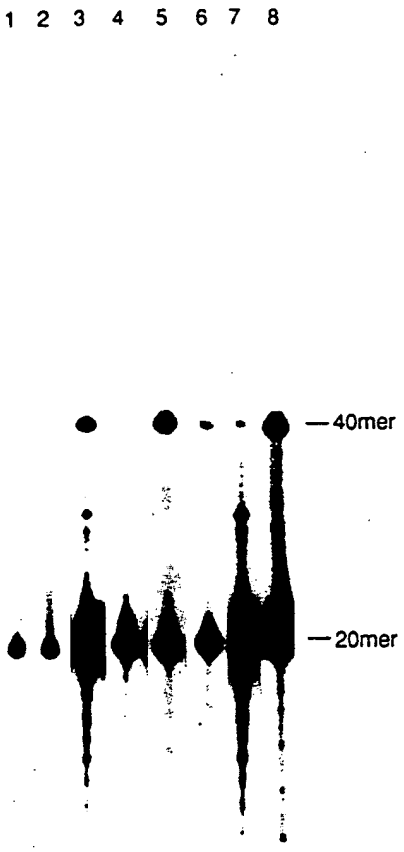


Fig. 4. Ligation reactions utilizing ss non-complementary oligos in the absence of any complementary target sequence. Oligos (lanes): (1) 88–100.1 and 88–101.2; (2) 88–139.1 and 88–140.1; (3) 88–101.2 and 88–100.1; (4) 88–101.2 and 88–100.1, without *Ec* ligase; (5) 88–99.1 and 88–102; (6) 88–99.1 only; (7) 88–101.2; and (8) 88–101.1 and 88–101.2, T4 DNA ligase. Each lane contains products resistant to AP treatment and was resolved in a 15% PA sequencing gel containing 8 M urea.

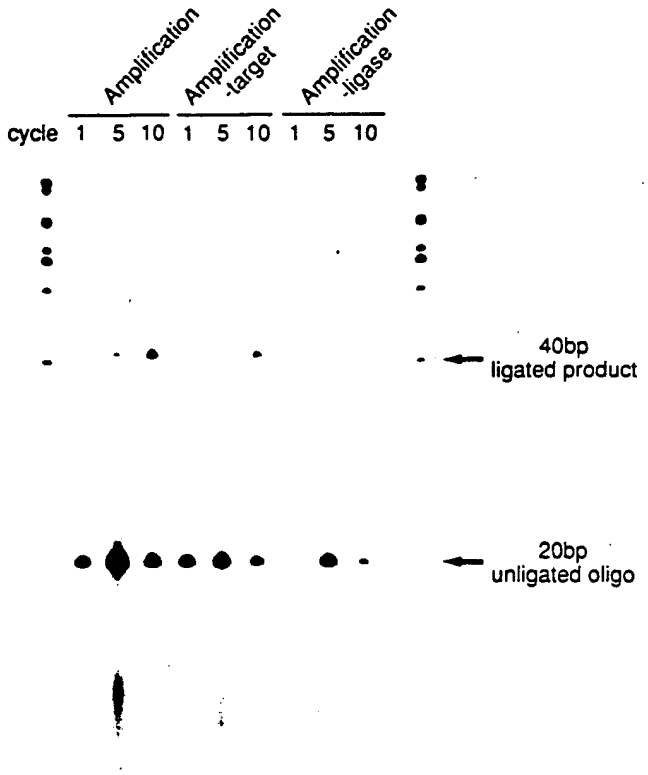


Fig. 5. Ligation products obtained from LAR reactions. LAR reactions were performed by mixing in equal molar amounts of 15 pmol of ^{32}P-labeled 20-nt donor oligos (88–101.2 and 88–139.1) with acceptor oligos (88–100.1 and 88–140.1), along with 0.1 fmol of a complementary target 40-nt oligo, in a total volume of 25 μl. Reactions without the 40-nt target oligo (88–165) served as control reactions. Amplification reactions were first heated for 3 min at 100°C, centrifugated briefly to remove any condensation, diluted with an equal volume of 2 × T-buffer (see Fig. 2 legend) and annealed at 60°C for 5 min. The volumes were adjusted to 60 μl by the addition of 10 μl of 1 × T-buffer containing 1 u of *Ec* ligase and 0.3 mM β-NAD$^+$ (no internal control DNA was added), and incubated at 37°C for 10 min. Each succeeding amplification cycle was then performed in the presence of oligos complementary to 88–100.1 and 88–101.2 by repeating the boiling and annealing steps as described above and replenishing the ligase and co-factor in 5 μl of 1 × T-buffer. Samples were removed after one, five or ten cycles, AP-treated, and resolved in a non-denaturing, 15% polyacrylamide gel.

ing the *Ec* ligase enzyme activity to 1 u. Consequently, we were encouraged to employ these conditions in a 10-cycle LAR reaction in an effort to increase the copy number of 0.1 fmol of a 40-bp target sequence. Aliquots of the LAR reaction were removed after one, five and ten cycles. As can be observed in Fig. 5, a 40-bp, AP-resistant ligation product was visible after the fifth cycle in a target-dependent reaction and after the tenth cycle in a target-independent reaction. No ligation products were observed in the absence of ligase. An eight- and 80-fold increase in the copy number of the target sequences was measured after the fifth and tenth cycles, respectively, of the target-dependent amplification. However, at the ten-cycle step, a 40-bp product was also observed in the target-independent reaction, which is approximately 36% of the signal noted for the target-dependent reaction.

(e) Conclusions

(1) In addition to the canonical nick-closing activity, both ss ligation and blunt-end ligation in the absence of volume-exclusion agents have been observed for *Ec* ligase.

(2) These target-independent ligations have been shown to be sensitive to the concentration of substrate oligos and the amount of *Ec* ligase. The temperature and concentrations of β-NAD$^+$ did not affect the target-independent activity.

(3) Employing ligation reaction conditions designed to minimize the target-independent ligation activities of *Ec* ligase, an LAR protocol was carried out for ten cycles. The results indicated an exponential increase in the copy number of the target nucleic acid by a factor of about 40 (signal-to-noise ratio of 2:1). This is lower than the theoretical

1024-fold increase and indicates an average 73% efficiency per ligation cycle. While this per-cycle ligation efficiency is comparable to the per-cycle amplification efficiencies achieved with other in vitro amplification methods (PCR and TAS), because of the background observed in these experiments an increase in ligation efficiency to >95% would be necessary to achieve the levels of amplification achieved by other methods. The use of a heat-resistant ligase would assist in improving this signal-to-noise ratio since the efficiency of target-independent ligation (blunt-end or ss) reactions would be diminished due to destabilization of the ligatable ends at the higher temperatures. There clearly exists a tantalizing opportunity to employ an improved version of this LAR method in conjunction with the ligase-mediated detection of single-nt mismatches (Wu and Wallace, 1989b; Landegren et al., 1988; Alves and Carr, 1988). Such a strategy would permit the detection of biologically important mutations which occur only at very low copy numbers.

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